Long-Term Effects of Intravitreal Injection of GMP-Grade Bone-Marrow–Derived CD34$^+$ Cells in NOD-SCID Mice with Acute Ischemia-Reperfusion Injury

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Purpose. To determine long-term safety of intravitreal administration of good manufacturing practice (GMP)–grade human bone-marrow–derived CD34$^+$ cells in NOD-SCID (nonobese diabetic–severe combined immunodeficiency) mice with acute retinal ischemia-reperfusion injury, a model for retinal vasculopathy.

Method. Acute ischemia-reperfusion injury was induced in the right eye of adult NOD-SCID mice (n = 23) by transient elevation of intraocular pressure. Seven days later, 12 injured eyes and 5 normal contralateral eyes were injected each intravitreally with 5×10^4 CD34$^+$ cells isolated under GMP conditions from a healthy human donor bone marrow using an immunomagnetic cell isolation system. The remaining 11 injured eyes were not treated and served as controls. Mice were euthanized 1 day, 4 months, and 8 months later. Both eyes were enucleated and examined by immunohistochemical analysis and hematoxylin and eosin staining. Among mice followed for 8 months, electroretinography (ERG) was performed on both eyes before euthanization. All major organs were examined grossly and histologically after serial sectioning.

Results. Immunohistochemical staining 4 months after injection showed detectable CD34$^+$ cells in the retinal vasculature. ERG at 8 months after CD34$^+$ cell injection showed signals that were similar in untreated eyes. Histology of the enucleated eyes injected with CD34$^+$ cells showed no intraocular tumor or abnormal tissue growth after 8 months. Histologic analysis of all major organs showed no abnormal proliferation of human cells.

Conclusions. Intravitreal administration of GMP-grade human bone-marrow–derived CD34$^+$ cells appears to be well tolerated long-term in eyes with acute retinal ischemic injury. A clinical trial will start to further explore this therapy. (*Invest Ophthalmol Vis Sci.* 2012;53:986–994) DOI:10.1167/iovs.11-8833

Retinal vascular diseases, such as retinal vascular occlusion and diabetic retinopathy, remain a common cause of blindness despite therapies available to treat associated complications such as macular edema and retinal neovascularization. Bone marrow stem cell (BMSC) therapy is a new area of research that is being investigated as possible therapy for various ischemic and degenerative diseases.[1–5] A subpopulation of BMSCs, referred to as lineage negative in animals or CD34$^+$ stem cells in humans, appears to be recruited to sites of ischemia and injury and play an important role in tissue healing through release of trophic factors.[2,4–9] These adult stem cells have been investigated in clinical and preclinical trials as therapy for various ischemic or degenerative conditions because they are easily obtained. Infusion of autologous BMSCs into the coronary artery is being used in clinical trials in patients with recent myocardial infarct to minimize cardiac failure.[2,5] Numerous clinical trials that use adult BMSCs are currently ongoing.[10–15]

The use of intravitreal BMSCs to treat retinal disease has been explored.[4,16–20] This route of administration is appealing since it is easy and limited numbers of cells are needed. To date, BMSCs injected intravitreally after mechanical or laser retinal injury were found incorporated in the injured outer retina after a year.[16,17] Intravitreal autologous lineage negative BMSCs in mice with retinal degeneration preserved some photoreceptors.[4,18]

The use of BMSCs in treating retinal vascular disease was explored by Caballero and colleagues[20] using murine models of both acute and chronic retinal vascular pathology (ischemia-reperfusion injury and streptozotocin-induced diabetes, respectively). Intravitreal injection of human CD34$^+$ cells resulted in rapid incorporation of these cells into the retinal vasculature within hours of injection. The retinal vasculature of treated eyes appeared to be less damaged than untreated eyes short-term. No long-term studies were done.

The use of autologous intravitreal BMSCs to treat patients with vision loss from retinal diseases has been explored in two small pilot clinical trials outside the United States. Jonas et al.[21]

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Presented in part at the annual meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, Florida, May 2011.

Supported in part by Research to Prevent Blindness unrestricted departmental grant, National Eye Institute (NEI) Grants EY 007739, EY012601, and U01HL087366 (MBG), Foundation Fighting Blindness Career Development Award (DGT), and NEI Core Grant P30 EY12576. This work was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 RR-12088-01 from the National Center for Research Resources, National Institutes of Health.

Submitted for publication October 20, 2011; revised December 14, 2011; accepted December 28, 2011.

Disclosure: **S.S. Park**, None; **S. Caballero**, None; **G. Bauer**, None; **B. Shibata**, None; **A. Roth**, None; **P.G. Fitzgerald**, None; **K.I. Forward**, None; **P. Zhou**, None; **J. McGee**, None; **D.G. Telander**, None; **M.B. Grant**, None; **J.A. Nolta**, None

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TABLE 1. Summary of Treatment Randomization, Follow-up, and Studies Performed on All NOD-SCID Mice

Mouse	OD	OS	Follow-up Period	Studies Performed
1	I/R + CD34$^+$	Control + CD34$^+$	1 d	Immunohistochemistry
2	I/R + CD34$^+$	Control + CD34$^+$	1 d	Immunohistochemistry
3	I/R + CD34$^+$	Control + saline	2 mo	Leg tumor—PCR; organs histology
4	I/R + CD34$^+$	Control + CD34$^+$	4 mo	Immunohistochemistry
5	I/R + CD34$^+$	Control + CD34$^+$	4 mo	Immunohistochemistry
6	I/R + CD34$^+$	Control/saline	4 mo	Histology
7	I/R + CD34$^+$	Control/saline	4 mo	Histology
8	I/R + CD34$^+$	Control/saline	4 mo	Histology
9	I/R + CD34$^+$	Control	8 mo	ERG, histology
10	I/R + CD34$^+$	Control	8 mo	ERG, histology
11	I/R + CD34$^+$	Control/saline	8 mo	ERG, histology
12	I/R + CD34$^+$	Control/CD34$^+$	8 mo	ERG, histology
13	I/R	Control	1 d	Immunohistochemistry
14	I/R	Control	1 d	Immunohistochemistry
15	I/R	Control	4 mo	Immunohistochemistry
16	I/R	Control	4 mo	Immunohistochemistry
17	I/R	Control	4 mo	Histology
18	I/R	Control	4 mo	Histology
19	I/R	Control	4 mo	Histology
20	I/R	Control	5 mo	Dead, no tissue
21	I/R	Control	8 mo	ERG, histology
22	I/R	Control	8 mo	ERG, histology
23	I/R	Control/saline	8 mo	ERG, histology

I/R, ischemia-reperfusion injury.

injected mononuclear cells intravitreally in three patients with end-stage macular degeneration, glaucoma, or diabetic retinopathy. No adverse event was noted during the follow-up period ranging from 2 to 12 months, but no visual benefit was reported. Siqueira et al.[22] also used intravitreal autologous mononuclear cells in five eyes with advanced retinal degeneration. No adverse event was noted after 10 months. Two eyes had improvement in electroretinography (ERG).

Based on these encouraging preclinical and clinical data, it is important to further explore the use of intravitreal BMSCs as treatment for retinal diseases, including retinal vasculopathy. Isolation of CD34$^+$ cells for delivery into the eye might make this therapy more effective in a clinical trial.[20] Before conducting a clinical trial, the current preclinical studies were conducted at the request of the U.S. Food and Drug Administration (FDA) to determine whether there are any long-term ocular and systemic side effects of intravitreal good manufacturing practice (GMP)–grade CD34$^+$ BMSCs in a relevant in vivo model. NOD-SCID (nonobese diabetic–severe combined immunodeficiency) mice with acute ischemia-reperfusion injury were used as a model of acute retinal vasculopathy since diabetic retinopathy could not be induced in SCID mice (Grant MB, unpublished data, 2009).

METHODS

This study was conducted according to a protocol that was approved by the Institutional Animal Care and Use Committee at the University

TABLE 2. Quantitative PCR Analysis of Leg Sarcoma in Mouse 3, Showing the Presence of Mouse DNA but the Absence of Human DNA

Well	Sample Name	DNA Source	Primers	Detector	Task	Ct
C2				SYBR	NTC	Undetermined
C3	Standard 1	293 cells (human)	Human ERV	SYBR	Standard	20.8041
C4	Standard 2	293 cells (human)	Human ERV	SYBR	Standard	23.7691
C5	Standard 3	293 cells (human)	Human ERV	SYBR	Standard	27.1899
C11	Tumor-huERV	Tumor	Human ERV	SYBR	Unknown	Undetermined
D2				SYBR	NTC	Undetermined
D3	Standard 1	293 cells (human)		SYBR	Standard	20.6435
D4	Standard 2	293 cells (human)		SYBR	Standard	23.6809
D5	Standard 3	293 cells (human)		SYBR	Standard	27.2700
D11	Tumor-huERV	Tumor	Human ERV	SYBR	Unknown	Undetermined
E2				SYBR	NTC	37.2011
E3	Standard 1	293 cells (human)	Mouse GAPDH	SYBR	Unknown	37.5900
E4	Standard 2	293 cells (human)	Mouse GAPDH	SYBR	Unknown	36.1318
E5	Standard 3	293 cells (human)	Mouse GAPDH	SYBR	Unknown	35.8578
E11	Tumor-moGAPDH	Tumor	Mouse GAPDH	SYBR	Unknown	27.4748
F2				SYBR	Unknown	Undetermined
F3	Standard 1	293 cells (human)	Mouse GAPDH	SYBR	Unknown	38.0180
F4	Standard 2	293 cells (human)	Mouse GAPDH	SYBR	Unknown	36.6484
F5	Standard 3	293 cells (human)	Mouse GAPDH	SYBR	Unknown	36.2384
F11	Tumor-moGAPDH	Tumor	Mouse GAPDH	SYBR	Unknown	28.3147

of California Davis and was compliant with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals

Twenty-three NOD-SCID mice, 8 to 12 months of age at initiation of the study (Jackson Laboratory, Bar Harbor, ME), were bred and maintained at the Animal Facility at the University of California Davis. At study termination the animals were euthanized by an overdose of ketamine and xylazine (14 and 30 mg/kg, respectively) followed by a thoracotomy, at which time both eyes were removed and fixed in 4% (w/v) buffered paraformaldehyde for immunohistochemical or histologic analysis. All major organs (brain, lung, liver, heart, kidney, pancreas, spleen) were removed and examined grossly for the presence of tumor, then preserved in optimal cutting temperature (OCT) medium for frozen sections.

Acute Ischemia-Reperfusion Injury

Under inhalation anesthesia (isoflurane vapor), acute ischemia-reperfusion injury was induced in the right eye of 23 mice by entering the anterior chamber with a 30-gauge needle attached to an infusion line of sterile saline and elevating intraocular pressure (IOP) transiently for 2 hours (80–90 mm Hg by Tono Pen; Medtronic Solan, Jacksonville, FL) as previously described.[20] Retinal ischemia was confirmed by whitening of the iris and loss of red reflex. After 2 hours, the needle was removed to allow the IOP to normalize and the eye to reperfuse. The contralateral left eye served as the control.

$CD34^+$ BMSC Isolation

Bone marrow from a healthy human donor was obtained commercially (Lonza, Walkersville, MD) and $CD34^+$ cells were isolated under GMP conditions using commercial density-gradient media (Ficoll-Paque; GE Healthcare [formerly Amersham Biosciences], Buckinghamshire, UK), followed by an immunomagnetic cell isolation system (CliniMACS; Miltenyi Biotec, Cologne, Germany). The isolated cells were washed, subjected to validated state sterility assays (including endotoxin assay and Gram staining), and resuspended in PBS to a final concentration of 5×10^4 $CD34^+$ cells/μL.

Intravitreal Injection of $CD34^+$ Cells

Seven days after the acute ischemia-reperfusion injury when retinal capillary damage would be appreciated,[23] the mice were randomized to either intravitreal $CD34^+$ cell injection ($n = 12$) or no treatment ($n = 11$), as shown in Table 1. Twelve eyes with acute ischemia-reperfusion injury and 5 control contralateral normal eyes from 12 injured mice were injected each intravitreally with 5×10^4 $CD34^+$ cells (GMP-grade) suspended in 1 μL PBS (see preceding text for details of isolation). The contralateral eye of 5 remaining mice with ischemia-reperfusion injury was injected intravitreally with 1 μL PBS.

All animals were kept under inhalation anesthesia (described earlier) during the procedure. A sterile 5-μL syringe (Hamilton Co., Reno, NV) attached to a 32-gauge A-bevel needle was used for intravitreal injection.

Mice were euthanized 1 day, 4 months, and 8 months after intravitreal injection unless they were noted to be in poor health.

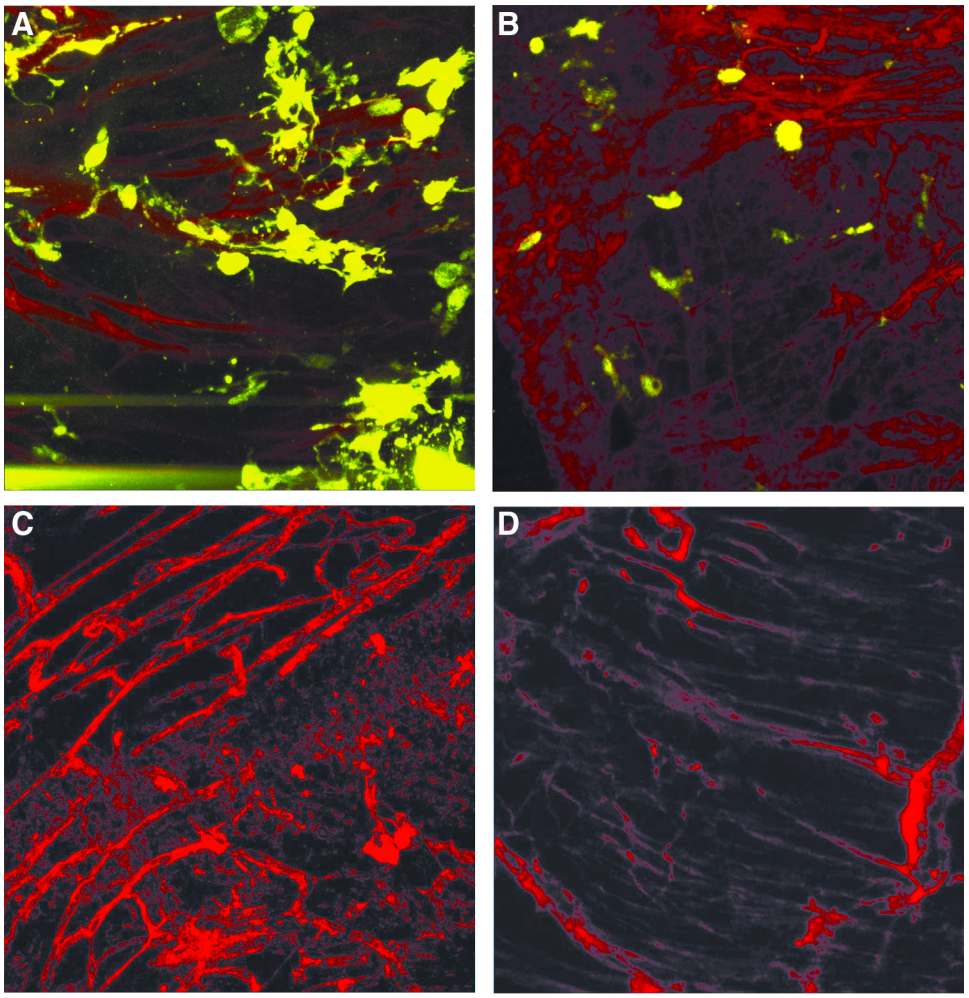

Figure 1. Immunohistochemical staining of retinal whole mount for human cells 1 day after intravitreal $CD34^+$ cell injection shows human cells (*green*) homing into the retinal vasculature of the NOD-SCID mouse eye with ischemia-reperfusion injury (**A**, mouse 1, right eye) more dramatically than that in the contralateral normal eye (**B**). Immunohistochemical staining of retinal whole mount of a NOD-SCID mouse untreated after ischemia-reperfusion injury shows a lack of detectable human cells in the ischemic eye (**C**, mouse 13, right eye) and the contralateral normal left eye (**D**).

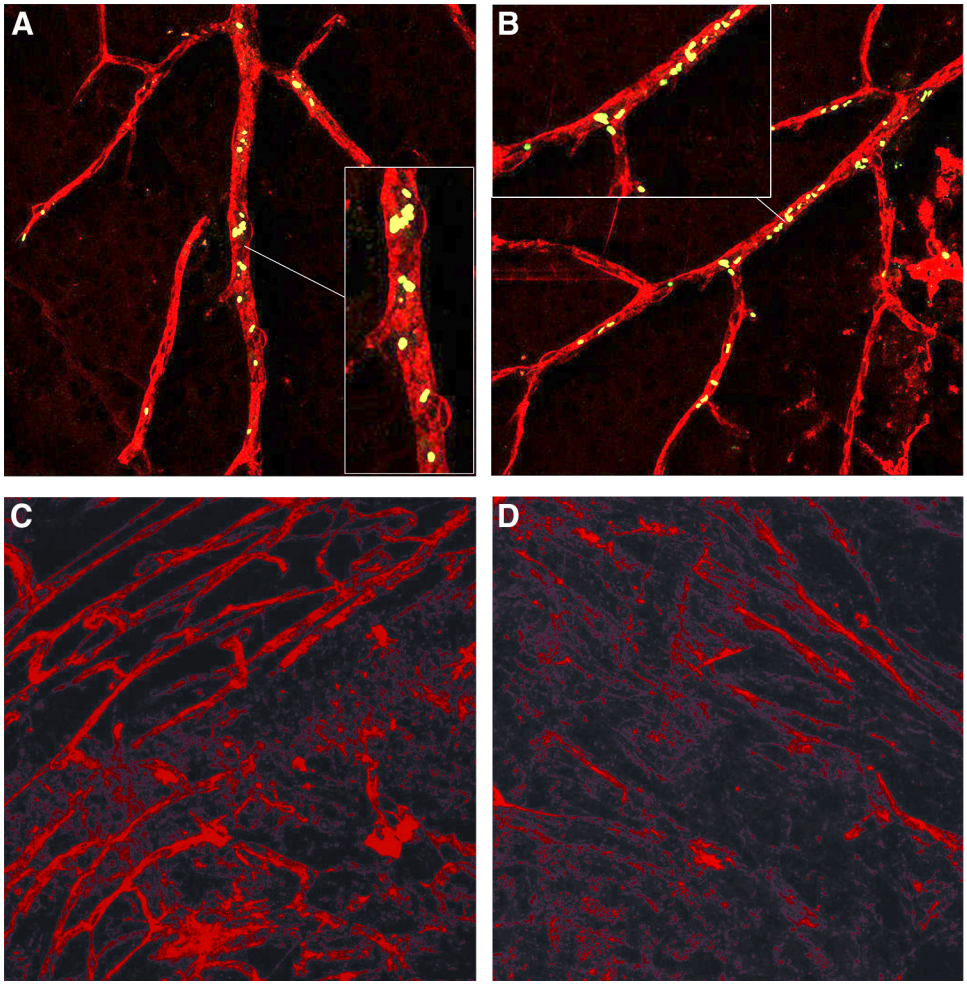

FIGURE 2. Immunohistochemical staining of retinal whole mount perfused with rhodamine-conjugated dextran to examine retinal vessel patency showing incorporation of human cells (*green*) into a normal-appearing retinal vasculature in an eye 4 months after acute ischemia-reperfusion injury and intravitreal injection of human CD34$^+$ cell injection (**A**, mouse 4, right eye). The contralateral normal eye injected with CD34$^+$ cells also shows detectable human cells incorporated in the retinal vasculature after 4 months (**B**). Immunohistochemical staining of retinal whole mount of NOD-SCID mouse eye untreated 4 months after ischemia-reperfusion injury (**C**, mouse 15, right eye) shows a lack of detectable human cells (*green*) in the retinal vasculature. A similar finding is noted in the normal untreated contralateral eye (**D**, mouse 15, left eye). *Insets* in (**A**) and (**B**) represent images magnified ×2 of the region of the retinal vasculature denoted.

Electroretinography

Among mice followed for 8 months, an ERG was performed on both eyes just before euthanasia. Mice were dark adapted overnight just before ERG testing (UTAS–EPIC XL; LKC Technologies, Gaithersburg, MD). Mice were then anesthetized intraperitoneally with a 0.1 mL/10 g dose of a ketamine (100 mg/mL to 1 mL) and xylazine (100 mg/mL to 0.1 mL) cocktail diluted 1:10 in sterile saline. After administration of anesthesia, mice were placed on a heating pad set at 38°C and both eyes dilated with 1% tropicamide and 2.5% phenylephrine. Proparacaine eye drops were applied for topical anesthesia. The eyes were lubricated with 1% methylcellulose. Mouse contact lens electrodes

TABLE 3. Immunohistochemical Analysis for the Presence of CD34$^+$ Cells in Retinal Vasculature

Mouse	Duration of Follow-up	Treatment Right Eye	CD34$^+$ Cells in Right Eye	Treatment Left Eye	CD34$^+$ Cells in Left Eye
1	1 d	I/R + CD34$^+$	+++	Control + CD34$^+$	+
2	1 d	I/R + CD34$^+$	+++	Control + CD34$^+$	+
4	4 mo	I/R + CD34$^+$	+	Control + CD34$^+$	+
5	4 mo	I/R + CD34$^+$	+	Control + CD34$^+$	+
13	1 d	I/R	ND	Control	ND
14	1 d	I/R	ND	Control	ND
15	4 mo	I/R	ND	Control	ND
16	4 mo	I/R	ND	Control	ND

I/R, ischemia-reperfusion injury; ND, nondetectable signal.

(LKC Technologies) were then placed on each eye, needle reference electrodes (LKC Technologies) were placed in each cheek, respectively, and finally a ground needle electrode was placed at the base of the tail. ERGs were generated with the following program: scotopic blue filter (0 dB) at 20 μV/div single flash; scotopic white (0 dB) at 50 μV/div single flash; photopic white (0 dB) 10 μV/div single flash; and photopic white (0 dB) 20 μV/div flicker, average of 10. After recording, the animals were euthanized as described earlier.

Immunohistochemistry of Retinal Vasculature

After removal, all eyes for immunohistochemical analysis were perforated with a 30-gauge needle and immersion fixed in 4% (w/v) buffered paraformaldehyde for 45 minutes, then washed in three changes of PBS. The procedure for immunohistochemical analysis was similar to a method previously described.[20]

The eyes were dehydrated in 2.5 M sucrose and then embedded in OCT medium for cryosectioning. At least 50 sections (10 μm thickness, every tenth section kept) were collected, and then postfixed in acetone for 5 minutes. The sections were then immersed in freshly prepared $NaBH_4$ (1 mg/mL in PBS) to reduce background autofluorescence, and then blocked in PBS containing 2% (w/v) nonfat dry milk and 2% (w/v) BSA. The sections were then reacted (with appropriate washes between incubations) with monoclonal rat anti-human CD31 (Abcam, Cambridge, MA) and monoclonal mouse anti-endothelium (Clone PAL-E; Abcam), both diluted 1:100 in PBS containing 1% (w/v) nonimmune rabbit serum and 1% nonimmune goat serum, followed by rhodamine-conjugated rabbit anti-rat IgG (Abcam) and FITC-conjugated goat anti-mouse IgG (Abcam), both diluted 1:200 in PBS. Sections incubated without primary antibody, but with secondary antibody, were used as controls. The slides were then mounted with commercial antifade medium (Vectashield; Vector Laboratories, Burlingame, CA) and digital image captures were made with an epifluorescence microscope (Zeiss Axioplan 2; Carl Zeiss, Inc., Thornwood, NY) coupled to a charge-coupled device camera (SPOT Imaging Solutions, Division of Diagnostic Instruments, Inc., Sterling Heights, MI).

Neural retinas from all the remaining eyes were dissected and permeabilized by overnight immersion in detergent buffer (10 mM HEPES, 150 mM NaCl, 0.2% [v/v] Triton X-100, 2% BSA, pH 8) at 4°C. Eyes from animals that were not perfused were then reacted with rhodamine-conjugated *R. communis* agglutinin I (1:1000 in 10 mM HEPES, pH 8; Vector Laboratories) to detect vasculature. The whole retinas were then mounted flat with antifade medium and digital image captures were made with a laser scanning confocal microscope (LSCM, BioRad MRC 1024; BioRad, Temecula, CA) and with an epifluorescence microscope (Zeiss Axioplan 2). ImageJ software (ImageJ 1.37n, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) was used for analysis of the confocal images.

Histology of Eye and Major Organs

Both eyes were removed and perforated with a 30-gauge needle and fixed with 4% (w/v) buffered paraformaldehyde. Every tenth section (10 μm thickness) of the whole eye was stained with hematoxylin and eosin (H&E). All major organs (see preceding text) were embedded in OCT medium for cryosectioning. Every tenth section (10 μm thickness) was collected and stained with H&E. Stained slides were analyzed under light microscopy by an eye pathologist (AR) who was blinded to the treatment conditions. If any abnormal tumor was noted, fluorescence in situ hybridization (FISH) or polymerase chain reaction (PCR) was performed to determine whether the cells were of human or murine origin.

FISH Analysis

Frozen sections of organs with microscopic evidence of tumor were further analyzed with FISH to determine the cellular origin of the tumor. Frozen tissue section slides were costained with a pan-centromeric chromosome probe from either mouse (green) or human (red) according to the manufacturer's specifications (Cambio Ltd, Cambridge, UK), with the following minor modifications as previously described.[24] Slides were incubated for 20 minutes on 10 ng/mL proteinase K. To denature DNA, slides were immersed in 70% formamide in 2 $\times$ SSC at 66°C for 5 minutes.

PCR and Quantitative Real-Time PCR

Large tumors that were grossly visible were analyzed by PCR to determine whether the tumor cells were of human or murine origin using a protocol previously described.[24] Mouse tumor cells were mechanically dissociated. Genomic DNA was extracted from these cells as described.[25] Amplification of the human *alu* gene via PCR using primers was done as described[26] under the following conditions: 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 45 seconds. Quantitative real-time PCR was performed using a commercial real-time PCR system under default conditions (ABI 7300; Applied Biosystems, Carlsbad, CA). The primers and probe for the human ERV-3 gene used were as previously described.[27] For the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, the following primers and probe were used: forward primer, accacgagaaatatgacaactaca; reverse primer, cccactgcctacataccatgagc; probe, 6FAM tcagcctgcatcctgcaccaccaact TAMRA. An absolute quantification standard curve for human or mouse was plotted using DNA from human cord blood mononucleated cells and nontransplanted mouse liver, respectively. The copy number was calculated. All samples and standards were assayed in duplicate reactions and averages were taken for calculation.

Table 4. Summary of Histopathologic Findings of Whole Eyes

Mouse	Duration of Follow-up	Treatment Right Eye	Findings Right Eye	Treatment Left Eye	Findings Left Eye
6	4 mo	I/R + $CD34^+$	Phthisis bulbi	Control/saline	Normal
7	4 mo	I/R + $CD34^+$	Normal	Control/saline	Normal
8	4 mo	I/R + $CD34^+$	Normal	Control/saline	Normal
9	8 mo	I/R + $CD34^+$	Total RD, ruptured lens capsule; no tumor	Control	Normal
10	8 mo	I/R + $CD34^+$	Normal	Control	Normal
11	8 mo	I/R + $CD34^+$	Normal	Control/saline	Normal
12	8 mo	I/R + $CD34^+$	Mild perineural inflammation	Control/saline	Mild perineural inflammation
17	4 mo	I/R	Phthisis bulbi	Control	Normal
18	4 mo	I/R	Normal	Control	Normal
19	4 mo	I/R	Normal	Control	Normal
21	8 mo	I/R	Phthisis bulbi	Control	Normal
22	8 mo	I/R	Normal	Control	Normal
23	8 mo	I/R	Normal	Control	Normal

I/R, ischemia-reperfusion injury.

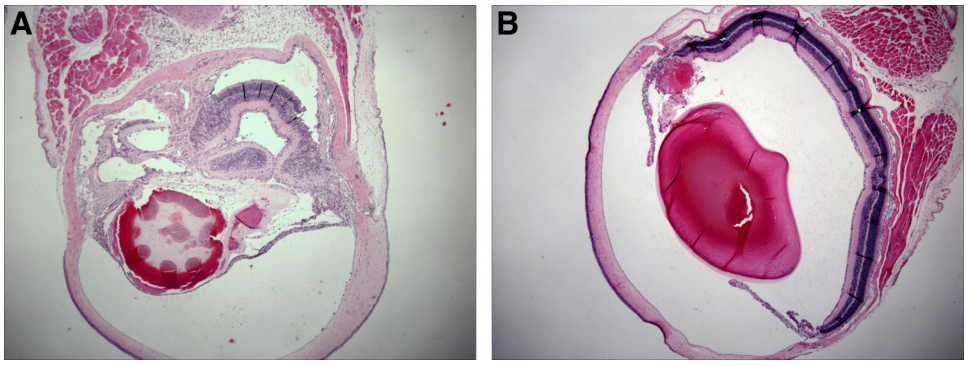

FIGURE 3. H&E-stained sections of whole eyes from NOD-SCID mice 8 months after ischemia-reperfusion injury and intravitreal injection of CD34$^+$ cells showing a range of histologic findings from chronic retinal detachment with disruption of the lens capsule (**A**, mouse 9, right eye) to that of a normal-appearing eye (**B**, mouse 10, right eye).

RESULTS

Table 1 summarizes the treatment randomization, the duration of follow-up, and outcome of the NOD-SCID mice used in this study. As shown, ischemia-reperfusion was induced in the right eye of all 23 mice. The left eye served as control. Among these 23 mice, 12 mice were injected with CD34$^+$ cells in the right eye 7 days after ischemia-reperfusion injury. Among these 12 mice, 5 had CD34$^+$ cells also injected in the contralateral normal left eye at the same time and another 5 mice had saline injected in the contralateral normal left eye. Among the 11 untreated mice, the contralateral eye was also untreated.

Mouse Survival during 8-Month Follow-up

During the 8-month course of this study, all mice survived to their randomized time point of the study except for one control mouse (mouse 20) that died at month 5; the organs could not be harvested for analysis. This mouse was not treated with CD34$^+$ cells. Another mouse (mouse 3) treated with CD34$^+$ cells in both eyes after ischemia-reperfusion injury in the right eye developed a visible leg tumor at month 2 and was euthanized early. H&E analysis of the leg tumor was consistent with a sarcoma. PCR analysis of the leg tumor revealed that the tumor cells contained DNA of mouse origin. No human DNA was found using the human ERV-3 gene primer and probe (Table 2). No other abnormality was noted in any of the other organs by H&E staining. We have previously described high rates of spontaneous murine tumors in immune-deficient mice, which may be due to a lack of natural killer function.[16] This tumor was unrelated to the treatment with human stem cells.

All remaining animals survived to the endpoint of the study. The study was terminated at month 8 since the average life expectancy of NOD-SCID mice is approximately 18 months.

Immunohistochemical Analysis of the Retinal Vasculature for CD34$^+$ Cells

Two mice were euthanized 1 day after intravitreal injection of CD34$^+$ cells for immunohistochemical analysis of the retinal vasculature (mice 1 and 2) and compared with untreated mice (mice 13 and 14). At this early time point, human cells were noted to have homed into the retinal vasculature much more prominently in eyes with retinal ischemia reperfusion injury when compared with the contralateral normal eye (Fig. 1). No CD34$^+$ cells were detected in untreated eyes.

When the same immunohistochemical study was performed in animals euthanized 4 months after the intravitreal injection of CD34$^+$ stem cells into both eyes (mice 4 and 5) and compared with eyes from untreated mice euthanized at the same time point (mice 15 and 16), human cells were detected incorporated into the retinal vasculature of both the eye with ischemia-reperfusion injury and in the contralateral normal eye but not detected in untreated eyes (Fig. 2). The findings are summarized in Table 3.

Histolopathologic Analysis of Whole Eyes

Table 4 summarizes the histolopathologic analysis of enucleated eyes harvested 4 and 8 months after ischemia-reperfusion injury. There were a total of 26 eyes from 13 mice. Among them, 7 mice had been treated with CD34$^+$ cells and 6 mice were untreated.

Among the seven mice injected intravitreally with CD34$^+$ cells after ischemia-reperfusion injury, one eye developed phthisis bulbi at month 4 (mouse 6) and a second mouse was noted with a total retinal detachment and disrupted lens capsule (mouse 9) after 8 months, with no intraocular tumor formation (Fig. 3A). Mouse 12 was noted with minimal inflammation around the optic nerve in both eyes. These histologic abnormalities were likely related to the trauma of the ischemia-reperfusion injury and/or intravitreal injection procedure in these small eyes rather than the presence of CD34$^+$ cells themselves, since all four remaining eyes in this group appeared unremarkable histologically (Fig. 3B). In particular, there was no abnormal proliferation of cells in the eye. These four eyes appeared histologically similar to the contralateral uninjured eye, which appeared normal in all mice.

TABLE 5. Summary of Electroretinography Findings 8 Months after Intravitreal CD34$^+$ Cell Injection

Mouse	Duration of Follow-up	Treatment Right Eye	ERG Signal Intensity Right Eye	Treatment Left Eye	ERG Signal Intensity Left Eye
9	8 mo	I/R + CD34$^+$	0	Control	+++
10	8 mo	I/R + CD34$^+$	++	Control	NA
11	8 mo	I/R + CD34$^+$	+	Control/saline	+
12	8 mo	I/R + CD34$^+$	+	Control/CD34$^+$	+++
21	8 mo	I/R	0	Control	NA
22	8 mo	I/R	++	Control	NA
23	8 mo	I/R	+	Control	+++

I/R, ischemia-reperfusion injury; NA, data not available since animal could not tolerate ERG testing in contralateral eye.

Among eyes not treated with $CD34^+$ cells after ischemia-reperfusion injury, two eyes developed phthisis bulbi after 4 months (mice 17 and 21) and a second had mild diffuse inflammation (mouse 21). All four remaining untreated injured eyes and all contralateral normal control eyes appeared unremarkable histologically. The findings were similar to those noted among eyes that had $CD34^+$ cells injected after ischemia-reperfusion injury.

Electroretinography

At 8 months after ischemia-reperfusion injury, ERG testing was performed in four mice treated with $CD34^+$ cells and three untreated mice. Table 5 summarizes the findings. ERG testing was attempted in both eyes in all animals, although the contralateral eye measurement was not possible in three mice since the animals did not tolerate the prolonged anesthesia required for bilateral testing and expired before the contralateral eye measurement could be obtained.

Among the seven ischemia-reperfusion injury–induced eyes, a detectable but possibly reduced ERG signal was noted in five of the eyes (Fig. 4A). The range of signal strength was similar among $CD34^+$ cell–treated eyes when compared with untreated eyes (Fig. 4B).

ERG was successfully recorded in the contralateral normal eye of only four of seven animals. All four had a detectable signal and three of the four recorded signals were strong. In particular, a normal eye that had $CD34^+$ cells injected had a strong, normal-appearing ERG signal after 8 months (Fig. 4C).

Histopathologic and FISH Analysis of Distant Organs

All major organs (brain, liver, lung, heart, kidney, spleen, pancreas) were harvested from all animals after 4 and 8 months; every tenth serial frozen section of the organ was stained with H&E and analyzed for the presence of tumor or other abnormalities. As summarized in Table 6, all major organs appeared unremarkable except for the liver of mouse 8, which showed the presence of a single microscopic nodule.

Frozen sections of this liver nodule were further analyzed by FISH. As shown in Figure 5, the liver section stained homogeneously positively with the mouse centromere probe (green) but did not stain with the human centromere probe (red).

Discussion

In this study, we used an acute ischemia-reperfusion model in NOD-SCID mice to study the long-term effect of intravitreal GMP-grade human $CD34^+$ cells from bone marrow in an eye with acute retinal vascular injury. This model is a well-established model to study the short-term effects of acute retinal ischemia.[20] However, the usefulness of this model in studying the long-term effect of retinal ischemia has not been established. Unfortunately, to date, there is no other established murine model of chronic retinal vasculopathy except diabetic retinopathy; diabetic retinopathy could not be induced in SCID mice so far, likely due to the immunodeficient state of the mouse (Grant MB, unpublished data, 2009).

Despite the limitations of this animal model, our study showed no major safety concerns of intravitreal GMP-grade $CD34^+$ cells. Although one mouse expired and did not survive to the endpoint of our study, this mouse was not treated with $CD34^+$ cells. Among the mice that were treated with $CD34^+$ cells, one mouse developed a leg sarcoma and a second mouse had a microscopic liver nodule on histologic analysis. However, both tumors were of murine origin and were not caused by proliferating human cells.

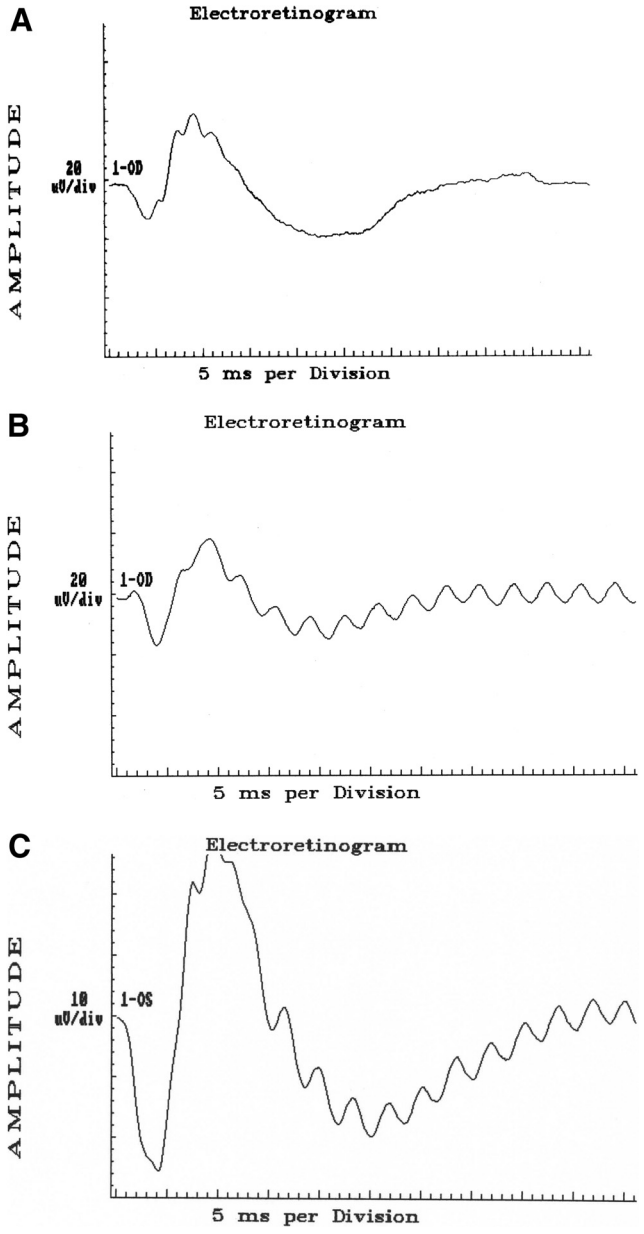

Figure 4. Electroretinogram from NOD-SCID mouse eyes 8 months after ischemia-reperfusion injury and intravitreal injection of $CD34^+$ cells shows a possibly reduced but detectable retinal signal (**A**, mouse 10, right eye), which is similar to that noted in an untreated eye 8 months after ischemia-reperfusion injury (**B**, mouse 22, right eye). ERG signal obtained from a normal eye injected with $CD34^+$ cells 8 months earlier (**C**, mouse12, left eye) shows a strong normal-appearing retinal signal.

On histologic analysis of the injected eyes 4 and 8 months after intravitreal injection of $CD34^+$ cells, no local safety concerns were noted. Two eyes did develop phthisis bulbi, lens disruption, and/or retinal detachment (mice 6 and 9). However, these changes were likely due to the trauma and damage induced by the ischemia-reperfusion injury, since similar histologic changes were also noted in two untreated eyes after ischemia-reperfusion injury (mice 17 and 21). What is interesting and surprising is that the majority of the eyes with acute ischemia-reperfusion injury appeared relatively normal on histopathology at 4 and 8 months after the injury, irrespective of whether the eye was treated with $CD34^+$ cells. In addition, the retinal vasculature at 4 months after intravitreal $CD34^+$ cells

TABLE 6. Summary of Histopathologic Findings of Distant Organs

Mouse	Duration of Follow-up	Treatment Right Eye	Treatment Left Eye	Findings of Distant Organs*
6	4 mo	I/R + CD34$^+$	Control/saline	Normal
7	4 mo	I/R + CD34$^+$	Control/saline	Normal
8	4 mo	I/R + CD34$^+$	Control/saline	Microscopic liver nodule–murine cells
9	8 mo	I/R + CD34$^+$	Control	Normal
10	8 mo	I/R + CD34$^+$	Control	Normal
11	8 mo	I/R + CD34$^+$	Control/saline	Normal
12	8 mo	I/R + CD34$^+$	Control/saline	Normal
17	4 mo	I/R	Control	Normal
18	4 mo	I/R	Control	Normal
19	4 mo	I/R	Control	Normal
21	8 mo	I/R	Control	Normal
22	8 mo	I/R	Control	Normal
23	8 mo	I/R	Control	Normal

* H & E staining of every tenth serial section of brain, lung, heart, liver, pancreas, spleen, and kidney.
I/R, ischemia-reperfusion injury.

appears relatively normal (Fig. 2A), suggesting that the eye can recover from the acute ischemic damage. Perhaps in untreated eyes, this recovery may be by the mice recruiting their own stem cells from the bone marrow as part of the normal healing mechanism.

ERG findings in our report, however, show that the retinal function may not have fully recovered from the ischemic insult despite the relatively intact-appearing histology. As shown in Table 5, the ERG signal in the injured eye was consistently decreased when compared with the contralateral normal eye. In two of seven eyes, the signal was nondetectable. However, theses two eyes had phthisis bulbi on histopathologic analysis. The range of signals in the ischemia-induced eye was similar irrespective of whether the eye was treated with CD34$^+$ cells, thus showing no dramatic improvement in retinal function with the treatment in this small study. However, although safety was demonstrated, the study was not designed to draw any significant conclusions about the efficacy of this therapy long-term.

What is encouraging is that mouse 12 had CD34$^+$ cells injected in the contralateral normal eye 8 months earlier and had a strong normal-appearing ERG signal (Fig. 4C), suggesting no adverse effect of the therapy itself on normal retinal function long-term. Similarly, among the injured eyes that were treated, the ERG signal range was similar to that of the untreated injured eyes, again supporting the hypothesis that there is no long-term toxic effect of this therapy on the retina.

In summary, intravitreal GMP-grade CD34$^+$ cells from bone marrow appear to be well-tolerated long-term in NOD-SCID mouse eyes with acute ischemia-reperfusion injury and normal eyes. This long-term preclinical safety information was submitted to the FDA and resulted in approval to start the first clinical study to explore the use of intravitreal autologous CD34$^+$ BMSCs to treat eyes with retinal vasculopathy.

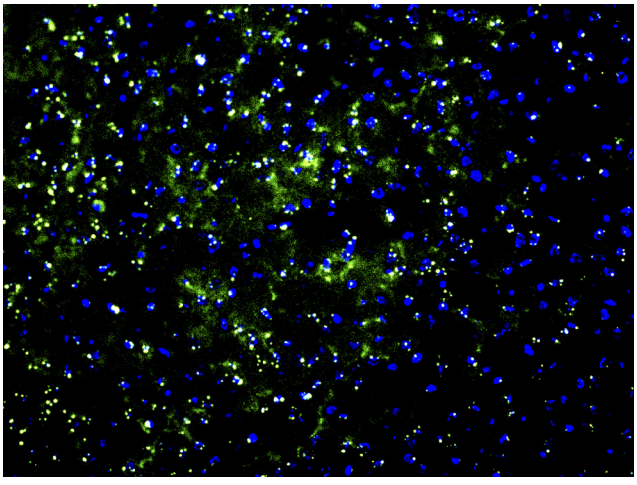

FIGURE 5. Fluorescence in situ hybridization of the liver frozen section from mouse 8 shows a homogeneous positive staining with the mouse centromere probe (*green*) but no staining with the human centromere probe (*red*).

References

1. Schachinger V, Zeiher AM. Stem cells and cardiovascular and renal disease: today and tomorrow. *J Am Soc Nephrol.* 2005;16:S2–S6.
2. Strauer BE, Brehm M, Zeus T, et al. Repair of infracted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation.* 2002;106:1913–1918.
3. Jones DG, Anderson ER, Galvin KA. Spinal cord regeneration: moving tentatively towards new perspectives. *NeuroRehabilitation.* 2003;18:339–351.
4. Otani A, Dorrell MI, Kinder K, et al. Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells. *J Clin Invest.* 2004;114:765–774.
5. Cleland JG, Freemantle N, Coletta AP, Clark AL. Clinical trials update from the American Heart Association: REPAIR-AMI, ASTAMI, JELIS, MEGA, REVIVE-II, SURVIVE, and PROACTIVE. *Eur J Heart Fail.* 2006;8:105–110.
6. Kang HJ, Kim MK, Kim MG, et al. A multicenter, prospective, randomized, controlled trial evaluating the safety and efficacy of intracoronary cell infusion mobilized with granulocyte colony-stimulating factor and darbepoetin after acute myocardial infarction: study design and rationale of the "MAGIC cell-5-combination cytokine trial" (Abstract). *Trials.* 2011;12:33.
7. Zohlnhofer D, Ott I, Mehilli J, et al. Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial. *JAMA.* 2006;295:1003–1010.
8. Harris JR, Brown GA, Jorgensen M, et al. Bone marrow–derived cells home to and regenerate retinal pigment epithelium after injury. *Invest Ophthalmol Vis Sci.* 2006;47:2108–2113.
9. Li Y, Reca RG, Atmaca-Sonmez P, et al. Retinal pigment epithelium damage enhances expression of chemoattractants and migration of bone marrow–derived stem cells. *Invest Ophthalmol Vis Sci.* 2006;47:1646–1652.
10. Satija NK, Singh Vk, Verma YK, et al. Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine. *J Cell Mol Med.* 2009;13:4385–4402.
11. Benoit E, O'Donnell TF Jr, Iafrati MD, et al. The role of amputation as an outcome measure in cellular therapy for critical limb ischemia: implications for clinical trial design. *J Transl Med.* 2011;9:Art. 165.

12. Lunn JS, Sakowski SA, Federici T, Glass JD, Boulis NM, Feldman EL. Stem cell technology for the study and treatment of motor neuron diseases. *Regen Med.* 2011;6:201–213.
13. Menasche P. Cardiac cell therapy: lessons from clinical trials. *J Mol Cell Cardiol.* 2011;50:258–265.
14. Joyce N, Annett G, Wirthlin L, Olson S, Bauer G, Nolta JA. Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regen Med.* 2010;5:933–946.
15. Meyerrose T, Olson S, Pontow S, et al. Mesenchymal stem cells for the sustained in vivo delivery of bioactive factors. *Adv Drug Deliv Rev.* 2010;62:1167–1174.
16. Tomita M, Adachi Y, Yamada H, et al. Bone marrow-derived stem cells can differentiate into retinal cells in injured rat retina. *Stem Cells.* 2002;20:279–283.
17. Keizo M, Yasushi A, Haruhiko Y, et al. Long-term survival of bone marrow-derived retinal nerve cells in the retina. *Neuro Report.* 2005;16:1255–1259.
18. Otani A, Dorrell MI, Kinder K, et al. Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells. *J Clin Invest.* 2004;114:765–774.
19. Smith LEH. Bone marrow-derived stem cells preserve cone vision in retinitis pigmentosa. *J Clin Invest.* 2004;114:755–757.
20. Caballero S, Sengupta N, Afzal A, et al. Ischemic retinal vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. *Diabetes.* 2007;56:960–967.
21. Jonas JB, Witzens-Harig M, Arseniev L, Ho AD. Intravitreal autologous bone-marrow-derived mononuclear cell transplantation. *Acta Ophthalmologica.* 2010;88:e131–e132.
22. Siqueira RC, Messias A, Voltarelli JC, Scott IU, Jorge R. Intravitreal injection of autologous bone marrow-derived mononuclear cells for hereditary retinal dystrophy: a phase I trial. *Retina.* 2011;31:1207–1214.
23. Zheng L, Gong B, Hatala DA, Kern TS. Retinal ischemia and reperfusion causes capillary degeneration: similarities to diabetes. *Invest Ophthlamol Vis Sci.* 2007;48:361–367.
24. Zhou P, Hohm S, Olusanya Y, Hess DA, Nolta J. Human progenitor cells with high aldehyde dehydrogenase activity efficiently engraft into damaged liver in a novel model. *Hepatology.* 2009;49:1992–2000.
25. Laird PW, Zijderveld A, Linders K, Rudnicki MA, Jaenisch R, Berns A. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* 1991;19:4293–4298.
26. Fukuchi Y, Miyakawa Y, Kizaki M, et al. Human acute myeloblastic leukemia-ascites model using the human GM-CSF- and IL-3-releasing transgenic SCID mice. *Ann Hematol.* 1999;78:2223–2231.
27. Yuan CC, Miley W, Waters D. A quantification of human cells using an ERV-3 real time PCR assay. *J Virol Methods.* 2001;91:109–117.